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The mouse IAP-promoted placental gene (MIPP) is ectopically expressed in BALB/c mouse mammary carcinomas, making it a candidate oncogene. One purpose of the proposed research, therefore, is to show whether MIPP, as it appears in the mammary tumors, is oncogenic. Determining the function of MIPP and if the solo long terminal repeat (LTR) associated with the gene promotes its ectopic expression are also proposed. Finally, the proposed research includes studying the human homolog of MIPP (IPP) in breast cancers. Using RT-PCR, we have shown that MIPP transcripts in mammary tumors do not contain LTR sequences, and therefore are not promoted by the solo LTR. This experiment also showed that all MIPP transcripts contain the same 3' end. Degenerate oligonucleotide-primed RT-PCR was used to amplify and clone part of the 5' region of MIPP from mammary tumors. A single open reading frame (orf), continuous with the orf at the 3' end, was found. This orf can code for an N-terminal BTB/POZ protein/protein interaction domain and six C-terminal *kelch* repeats. Therefore, MIPP may be an actin-binding protein. RT-PCR analysis of normal and malignant human breast cell lines showed that IPP is not expressed in these cells.

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John M. Van Hoof 7/24/97

PI - Signature Date

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INTRODUCTION:

Intracisternal A particles (IAPs) are retrotransposons of which 1000 copies are endogenous to the haploid mouse genome (1). The full-length IAP element is 7.2 kb, although several classes of IAPs contain deletions and range in size from 5.4 to 2.9 kb. Like retroviruses, IAPs have gag, pol, and env sequences flanked on both ends by long terminal repeats (LTRs). However, the env gene of most IAPs is defective because it contains numerous stop codons, and consequently IAPs are not horizontally transmissible (2). The LTRs of IAPs carry the signals necessary for promotion, initiation, and polyadenylation of transcription (3). IAPs are abundantly expressed in many different types of mouse tumors and during development of the preimplantation embryo, but are usually not expressed in most normal adult tissues (2; 4).

Due to their ability to integrate into new sites in the genome and alter the expression of cellular genes, IAPs are a source of genetic variability and can contribute to neoplastic transformation (5). The insertion of IAPs has been found to activate cellular oncogenes such as c-*mos* (6; 7) and to cause constitutive expression of growth factors and growth factor receptors including interleukin-6 (8), interleukin-6 receptor (9), granulocyte macrophage colony stimulating factor (10) and interleukin-5 (11). IAP transposition triggered constitutive expression of interleukin-3 in a leukemia cell line (12), and in a separate incident, induced autocrine transformation by stabilizing interleukin-3 mRNA in hemopoietic cells (13).

IAPs can also regulate transcription of genes in normal cells. Germ-line retrotranspositions have linked IAPs to the thrombomodulin gene (14) and the angiotensinogen gene (15), but the effect, if any, of these IAPs on the transcription of these genes is not known. However, the oncomodulin gene in the rat (16) and the mouse IAP-promoted placental (MIPP) gene (17) are promoted by solo IAP LTRs in a tissue-specific manner. The presence of a solo LTR at the 5' end of the MIPP gene is presumed to have arisen by a germ line retrotransposition with subsequent loss of protein-coding IAP sequences via unequal crossing over at the LTRs. A solo intracisternal A-particle (IAP) long terminal repeat (LTR) present in the mouse MIPP gene promotes placenta-specific expression of a 1.2 kb message. This transcript is a truncated form of 2.2 and 4.4 kb MIPP transcripts also found in placental tissue (18). In the myeloma MOPC-315, which is known to express IAPs (19), the 2.2 kb MIPP transcript is highly expressed (17).

The MIPP gene appears to have an important function because it is evolutionally conserved. Sequences homologous to MIPP were detected in monkey, hamster, and human DNA by Southern blotting (17). The human homolog of MIPP, IPP, was assigned to chromosome 1p32-1p22 (20). The truncated 1.2 kb MIPP transcript codes for a putative protein of 202 amino acids, belonging to the *kelch* family of proteins (21). Several *kelch* family proteins have been shown to bind actin. However, the MIPP product has only been deduced from nucleic acid sequences and has not been directly identified or characterized to date.

We have previously shown that BALB/c mouse mammary preneoplasias and carcinomas of several etiologies ectopically express 2.2 and 5.6 kb MIPP-related mRNAs. MIPP messages were not detected in normal mammary gland from virgin, pregnant, and lactating mice. The 5.6 kb transcript appears to be unique to the mammary lesions. Additionally, in the mouse mammary tissues, IAP expression was found to correlate with MIPP expression.

The ectopic expression of MIPP in the mammary tumors makes it a candidate for a novel oncogene in BALB/c mice. Therefore, one of the purposes of the proposed research is to determine whether the two MIPP mRNA species expressed in BALB/c mammary carcinomas are oncogenic. This necessitates the cloning of these transcripts for use in transformation studies.

Use of MIPP cDNAs in an expression system will also be of value in isolating and determining the function of MIPP protein(s).

As suggested by the parallel expression of IAPs and MIPP in BALB/c mammary tissues, the solo LTR of MIPP may promote expression of the 2.2 and 5.6 kb transcripts in the tumors. If so, then either general activation of IAP LTRs or activation specifically of the MIPP LTR may be the mechanism by which MIPP expression is ectopically activated.

Since IPP has not been studied in human mammary epithelial cells or tissues, the proposed research will examine its expression in normal and malignant human breast cell lines and in human breast cancers. This will indicate whether IPP might be a human oncogene involved in breast cancer.

EXPERIMENTAL METHODS, ASSUMPTIONS, AND PROCEDURES:

1. To address the question of whether activation of the solo LTR causes MIPP to be expressed in BALB/c mouse mammary tumors, a reverse transcriptase-polymerase chain reaction (RT-PCR) strategy was used. For this experiment, I assumed that all MIPP mRNAs use the same polyadenylation signal. RNA from a MIPP-expressing BALB/c mammary tumor, from normal mammary gland of a lactating mouse, and from placenta were reverse transcribed using an oligo-dT primer and Moloney murine leukemia virus (MMLV) reverse transcriptase (RT). Two sets of PCR reactions were carried out on the cDNA from these three tissues, using a 3' primer derived from the region containing the polyadenylation signal. One set of reactions used a 5' primer from the MIPP LTR, and the other set used a 5' primer downstream from the LTR (Figure 1). This method will reveal whether the IAP LTR is present at the 5' end of MIPP transcripts, and therefore, might promote their transcription.

2. Several strategies have been employed with the aim of cloning the MIPP mRNAs found in BALB/c mammary carcinomas.

A. A cDNA library was constructed with poly-A^+ RNA from a D2 mammary tumor known to express MIPP and the ZAP-cDNA Gigapack II kit (Stratagene, La Jolla, CA). Oligo-dT was used to prime first strand cDNA synthesis by MMLV RT. RNaseH and *E. coli* DNA polymerase I were used for second strand synthesis. ^{32}P-dATP-labelled controls for first and second strand synthesis were analyzed by alkaline agarose gel electrophoresis. Following second strand synthesis, the cDNA was blunted using *Pfu* DNA polymerase, and two synthetic EcoRI adaptors were ligated to the cDNA with T4 DNA ligase. The cDNA was then digested with XhoI which cuts one of the adaptors so that cDNA can be directionally cloned. The cDNA was size selected on a sephacryl column for DNA greater than 300 base pairs (bp). After kinasing the cDNA, it was ligated into Uni-ZAP XR vector arms. The library was packaged using Gigapack II Gold packaging extract (Stratagene, La Jolla, CA) and plated on the *E. coli* cell line XL1-Blue MRF'. The library was amplified, titrated, and duplicate plate lifts were hybridized with the ^{32}P-dCTP-labelled MIPPd4 clone. A total of 1 x 10^6 plaque forming units were screened. As a second method of screening, PCR using a vector-specific primer combined with a MIPP-specific primer was performed.

B. The 1.2 kb MIPP transcript has an open reading frame (orf) capable of coding for a protein with four repeating IYALGG motifs. These repeats define members of the *kelch* family of proteins, and will be referred to as *"kelch* repeats." Some proteins in the *kelch* family also contain an N-terminal protein/protein interaction domain known as BTB/POZ. Assuming that the full-length MIPP cDNAs (2.2 and 5.6 kb) encode proteins with BTB/POZ domains, a degenerate primer was designed for RT-PCR (Figure 1B). The degenerate 5' primer was derived from the

most conserved area of the BTB/POZ domain-encoding sequence, and two 3' primers were made to sequences downstream from the MIPP LTR. Two rounds of PCR, a low stringency round followed by a higher stringency nested round, were carried out on oligo-dT-primed cDNA from a D2 tumor which expresses MIPP. The resulting DNA was electrophoresed on an agarose gel and the 950 bp band was excised and purified. A T-overhang vector, pGEM-T (Promega) was used to clone the PCR product. Manual sequencing by the dideoxynucleotide method confirmed the presence of the degenerate primer at one end and the nested 3' primer at the other. The entire clone was sequenced by the institute's Biopolymer/Sequencing facility (Figure 2).

C. Various 5' rapid amplification of cDNA ends (RACE) methods were applied to cDNA synthesized from a D2 tumor to obtain the far 5' ends of the 2.2 and 5.6 kb MIPP transcripts. In the first of these methods, a homopolymeric tail was added to single stranded cDNA using terminal transferase (Figure 3). Reverse transcription was primed with an oligonucleotide complementary to part of the cDNA cloned using the BTB/POZ degenerate primer/PCR technique. After removal of the RT primer, either a poly-A or poly-G tail was added. The tailed cDNA was then amplified by two rounds of PCR with the appropriate 5' primer and two nested gene-specific primers. The amplified DNA was then cloned into a T-overhang plasmid and sequenced.

Another technique used to clone the 5' ends of the MIPP mRNAs involved ligating a single-stranded oligonucleotide adapter to the single-stranded cDNA (Figure 3). The oligonucleotide was synthesized with a 3' amine and a 5' phosphate to ensure that it could only be ligated on to the 3' end of the first strand cDNA (which represents the 5' end of the RNA). T4 RNA polymerase was used to catalyze the ligation. After removal of excess adapter oligonucleotides, PCR was performed using a 5' primer complementary to the adapter and two nested gene-specific primers. Amplified DNA was purified, cloned using the T/A method, and sequenced.

A system related to the ligation-mediated PCR strategy described above, but using a double stranded adapter, was also used (Figure 3). Double stranded cDNA was made from the first strand cDNA with RNaseH and E. coli DNA polymerase I. The cDNA was then blunted with T4 DNA polymerase and was ligated to the double stranded oligonucleotide adapter with T4 DNA ligase. Nested PCR was then done using an adapter-specific primer and two downstream MIPP-specific primers. Resulting DNA fragments were T/A cloned and sequenced.

Finally, an inverse PCR strategy was designed to allow amplification of the 5' ends of the MIPP mRNAs in the mouse mammary tumors (Figure 4). Double stranded cDNA was made as above, and T4 DNA ligase was added with the intent of circularizing the cDNA. In a variation of the method, EcoRI adapters were ligated to the ends of the double-stranded cDNA, excess adapters were removed, and the cDNA was then circularized. The presumed-circular cDNA was then digested with AvaI, which cuts the cDNA once to linearize it. Primers specific for known MIPP RNA sequences which pointed away from each other on either side of the AvaI site would have been pointing towards each other in the linear cDNA. The 5' end of the cDNA should be between the primers, and amplifiable by PCR.

3. Expression of IPP, the human homolog of MIPP, was examined in a mortal primary culture of human mammary epithelial cells from a reduction mammoplasty (HMEC), in a benign immortal breast cell line (HBL100), and in breast cancer cell lines (MCF-7, MDA-MB-231, and T47D). The 3' end of IPP has been cloned by Chang-Yeh et al. (20), and contains a region 280 bp lone which is 83% homologous to the MIPP cDNA at the nucleotide level. Therefore, PCR primers were designed to amplify most of this homologous region, and mouse placenta was used

as a positive control. Total RNA from each of the cells or tissues was primed with random hexamers and reverse transcribed with MMLV RT. PCR was then carried out, and products were analyzed on 1% agarose gels (Figure 5).

RESULTS AND DISCUSSION:

1. The parallels between the expression of MIPP and IAPs suggested that the IAP LTR may play a role in transcriptional regulation of the gene. To determine whether the 2.2 kb and 5.6 kb transcripts might be promoted by the LTR, an RT-PCR strategy was used (Figure 1A). Using a 3' primer corresponding to a region near the polyadenylation signal, and 5' primer downstream from the U5 region of the LTR from the MIPP cDNA clone (17), one band of 881 bp was amplified from placenta and a D2 tumor. This band is the one expected to be amplified from the 1.2 kb placental transcript (17). However, using the same 3' primer with a 5' primer including 15 bases from the U5 sequences of the LTR, the anticipated band of 949 bp was amplified from placenta but no band was amplified from the D2 tumor. Therefore, LTR sequences are not present in the MIPP mRNA expressed in mouse mammary tumors. Furthermore, the size of the fragment amplified by the downstream (non-LTR) 5' primer was the same in the tumor as in placenta, and only one band was amplified from each tissue, despite the expression of multiple related transcripts. This indicates that all MIPP-related transcripts (1.2 kb, 2.2 kb, 4.4 kb, and 5.6 kb) have a common 3' end starting after the LTR. There are two explanations which account for both the absence of LTR sequences in the 2.2 kb and 5.6 kb transcripts, and the fact that they are longer than the 1.2 kb transcript only in the 5' direction. Since the LTR must lie between the common 3' end of the RNAs and the extended 5' ends of the 2.2 kb and 5.5 kb transcripts, the transcriptional machinery either skipped over the LTR during transcription of the larger transcripts, or it read through the stop site in the LTR, and the LTR was later spliced out. As expected, neither MIPP-specific primer set used in the RT-PCR amplified cDNA from normal mammary gland of a lactating mouse.

2. The high frequency of ectopic MIPP expression in both mouse mammary preneoplasias and tumors suggests that MIPP might be a new oncogene which contributes to at least some pathways of neoplastic progression in the BALB/c mouse mammary gland. To determine whether MIPP is a mammary oncogene, it is necessary to clone and characterize the transcripts expressed in the tumors. The effects of these clones on the *in vitro* and *in vivo* growth properties of mammary epithelial cells can then be analyzed. Therefore, a cDNA library was made from a MIPP expressing tumor and screened with a MIPP nucleic acid probe and by PCR. No positive clones were identified by either method.

The *kelch* family of proteins can be divided into two sub-families: those which contain a BTB/POZ protein/protein interaction domain, and those which do not. By virtue of its *kelch* repeats, the putative MIPP protein was deemed a member of the *kelch* family (21). However, since only a fragment of the MIPP mRNAs had been cloned, the division to which it belonged was not known. Therefore, a degenerate oligonucleotide complementing BTB/POZ sequences was used to PCR amplify cDNA from a MIPP-expressing mouse mammary tumor. A cDNA of about 994 bp was amplified, cloned and sequenced (Figure 1B, 2). The cDNA has an open reading frame continuous with that of the previously described 3' end (17). Sequence analysis of the cDNA revealed the presence of two additional *kelch* repeats at the 3' end, and a BTB/POZ domain at the 5' end. The untruncated predicted MIPP protein(s) are now known to contain a total of six *kelch* repeats, which is characteristic of most *kelch* proteins, and can be subdivided into the BTB/POZ division. The sum of known MIPP cDNA sequences is 1823 bp, leaving

approximately 200 bp and 3.6 kb of unknown sequences in the 2.2 and 5.6 kb transcripts, respectively (assuming a poly-A tail of 200 nucleotides).

The *kelch* family includes: the *Limulus* protein, scruin, the Drosophila *kelch* gene product, various vaccinia virus proteins, mammalian calicin, the nerve-cell-specific ENC1, and the Caenorhabditis elegans protein spe26 (22),. Scruin is an actin filament cross-linking protein in *Limulus* sperm (23), kelch associates with actin in structures known as ring canals during oogenesis in Drosophila (24), calicin is associated with actin in the acrosome of mammalian sperm (25), and ENC1 is an actin-binding protein found in nervous cells (26). Furthermore, it has been suggested that the vaccinia proteins in this family may mediate the effects of poxviruses on the host cells' actin cytoskeleton to facilitate their intracellular movement and infectivity (23; 27).

The actin cytoskeleton serves various functions, some of which are directly altered in the malignant cell phenotype (reviewed in 28). The actin filament network functions in determining cell shape and movement, mediates cytokinesis, and participates in cell-cell interactions and signal transduction pathways via associations with plasma membrane proteins. Disruption of these functions correlates well with derangements in growth behavior and morphology that characterize malignantly transformed cells. Indeed, disorganization of the actin cytoskeleton itself is often an attribute of transformed cells. Thus, it is tempting to speculate that the MIPP protein's function involves binding to actin, thereby contributing to neoplastic progression when ectopically expressed. However, since the entire MIPP cDNAs remain uncloned, direct evidence that MIPP proteins bind actin and/or are oncogenic is lacking.

Therefore, 5' RACE/PCR was used to amplify the 5' ends of the MIPP transcripts found in mouse mammary carcinomas. Methods involving addition of 5' homopolymer tails to cDNA, addition of either single- or double-stranded adapters to the 5' end of cDNA, and circularization of cDNA followed by inversion of opposite facing primers by digestion with a restriction endonuclease were all attempted (Figures 3, 4). For each technique, many variations in cDNA synthesis and PCR conditions were tried. However, the results varied from obtaining no amplification products whatsoever, to artefacts which proved to be unrelated to MIPP upon sequence analysis.

The difficulty encountered in cloning the 5' end of MIPP RNAs may be explained by the situation described by Hernandez *et al.* (26) regarding another *kelch*-related protein, ENC-1. In adult brain, a 4.5 kb ENC-1 transcript is expressed, but only the 3' end (2.4 kb) was cloned. The 2.4 kb clone had a large reading frame with a stop codon in a G/C rich area upstream from the translation start site. It was concluded that the rest of the 5' end must be untranslated (26). If a similar condition exists for the MIPP gene, then our difficulty cloning the 5' ends could be due to a high G/C content, since this may inhibit their reverse transcription.

3. IPP, the human homolog of MIPP, has 280 bp near its 3' end which are over 80% identical to the mouse gene at the nucleotide level (20). RT-PCR primers from this area were used to screen HMEC, HBL-100, MCF-7, MDA-MB-231, and T47D cells for IPP expression. Mouse placenta was included as a positive control for expression, and actin primers were used as a control for reverse transcription and amplification. Controls which excluded the RT enzyme were also done (data not shown). No product was obtained with human cells, indicating that none of them express IPP (Figure 5). Accordingly, it is probably not an oncogene in humans, although actual breast cancer tissues have not been analyzed.

RECOMMENDATIONS RELATED TO STATEMENT OF WORK (SOW):

Task number one, which was essentially to determine whether the LTR promotes MIPP transcription in the mouse mammary tumors, has been accomplished. However, alternative methods to those outlined in the SOW were used. Tasks two and three are to determine whether MIPP is an oncogene in mouse mammary carcinogenesis and to determine the function of MIPP protein(s), respectively. These tasks depend upon the cloning of the tumor-associated MIPP transcripts, which has been the major difficulty thus far. Using a degenerate oligonucleotide-primed PCR strategy, this has been partially accomplished. However, attempts to clone the far 5' ends of MIPP transcripts have been unsuccessful. Therefore, future research will continue to concentrate on cloning the 5' ends of MIPP RNAs. Progress has been made in accomplishing task four, analyzing IPP in human breast cancers. IPP expression was not detected in human breast cancer cell lines. However, analysis of breast cancer tissues for IPP expression and analysis of the IPP gene in DNA of breast cancers and breast cancer cell lines will still be done. These experiments are warranted because genes are frequently differentially expressed in cultured cancer cells and cancer tissues.

CONCLUSIONS:

We found that the IAP LTR in the MIPP gene does not promote the ectopic transcription of the 2.2 and 5.6 kb MIPP RNA species in BALB/c mouse mammary carcinomas. Accordingly, another mechanism must be responsible for activation of the MIPP gene in mouse mammary tumorigenesis. Elucidation of this mechanism will not be possible until the promoter of full-length MIPP transcripts is defined.

Additionally, we showed that IPP, the human homolog of MIPP, is not expressed in normal and malignant breast cells. Therefore, IPP may not be a breast cancer oncogene. Notwithstanding, IPP may yet be expressed in breast cancer tissue.

On the basis of sequence data, the putative MIPP protein encoded by the 2.2 and 5.6 kb transcripts can be classified as a BTB/POZ-containing *kelch* protein. Since most *kelch* proteins bind actin, the MIPP protein(s) probably also does. The presence of a BTB/POZ domain opens the possibility that MIPP can form homodimers, thereby cross-linking actin filaments, or that MIPP can form heterodimers, thus localizing actin filaments to other cellular structures. As is often the case, we may learn much about the function of a protein from its behavior in normal versus malignant cells.

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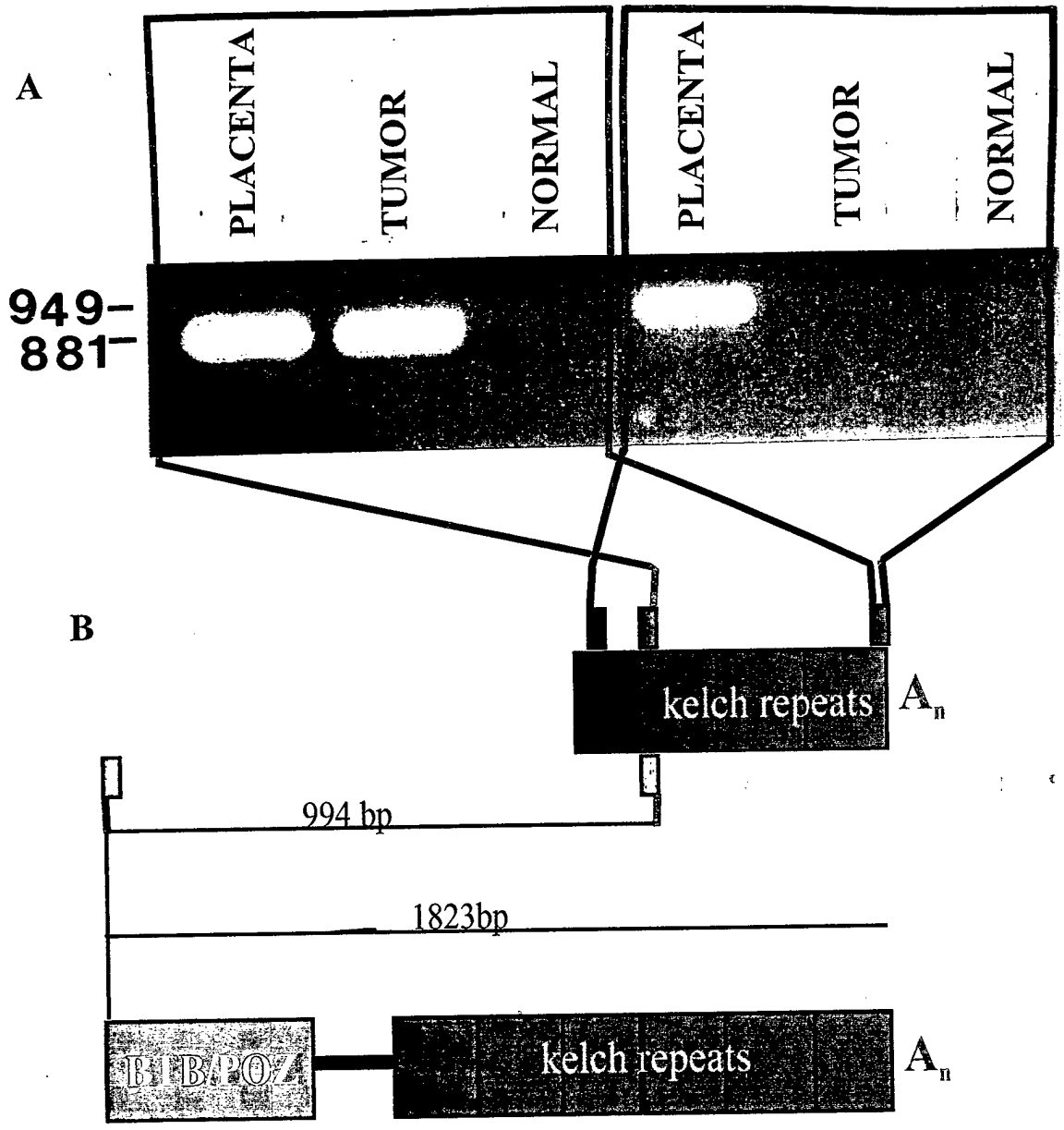
FIGURE 1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PLACENTA
TUMOR
NORMAL
PLACENTA
TUMOR
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881-
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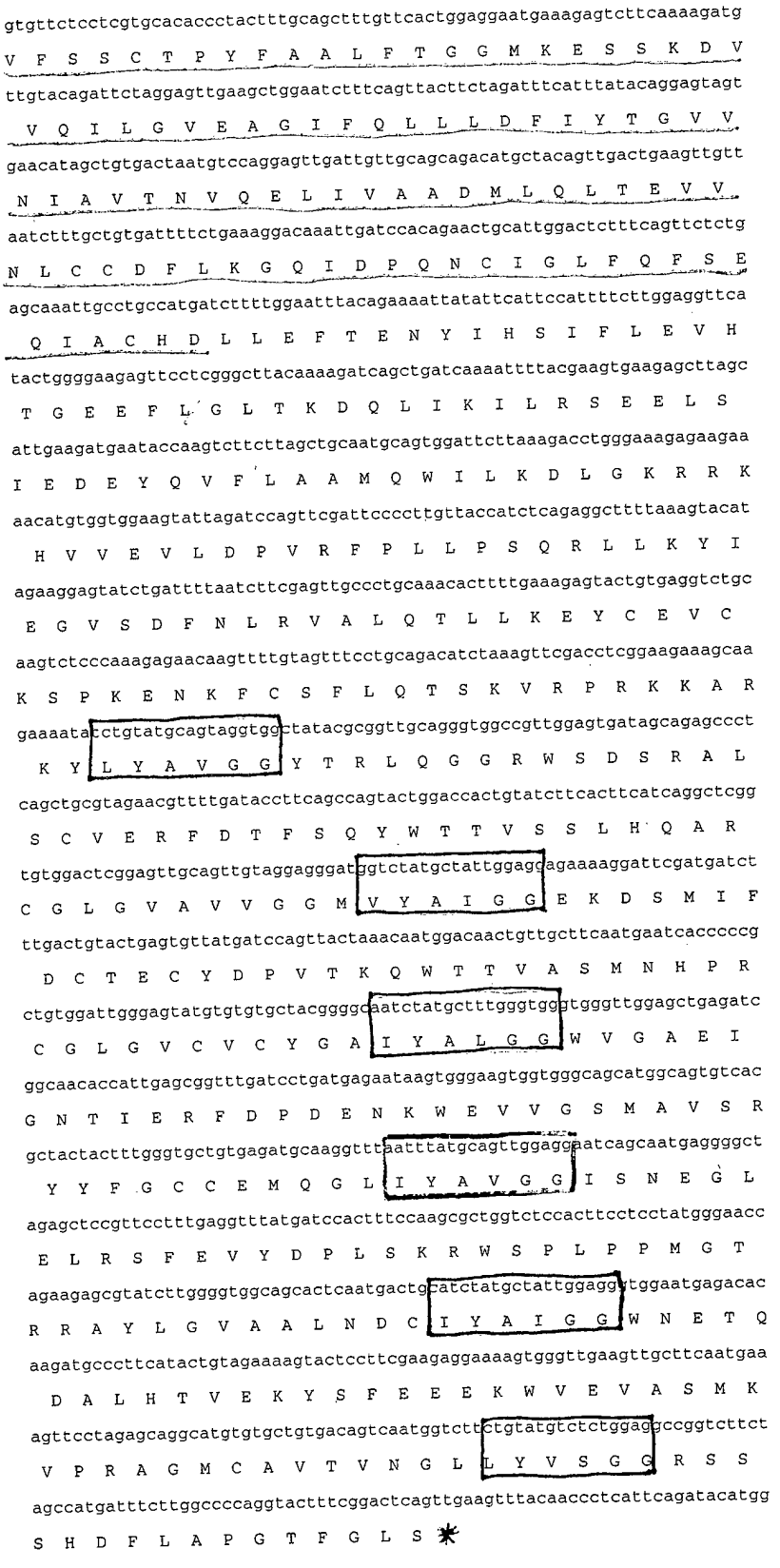
kelch repeats
A_n
994 bp
1823bp
kelch repeats
A_n

FIGURE 2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S C V E R F D T F S Q Y W T T V S S L H Q A R
tgtggactcggagttgcagttgtaggagggatggtctatgctattggagcagaaaaggattcgatgatct
C G L G V A V V G G M V Y A I G G E K D S M I F
ttgactgtactgagtgttatgatccagttactaaacaatggacaactgttgcttcaatgaatcacccccg
D C T E C Y D P V T K Q W T T V A S M N H P R
ctgtggattgggagtatgtgtgtgctacgggggcaatctatgctttgggtgggtgggttggagctgagatc
C G L G V C V C Y G A I Y A L G G W V G A E I
ggcaacaccattgagcggtttgatcctgatgagaataagtgggaagtggtgggcagcatggcagtgtcac
G N T I E R F D P D E N K W E V V G S M A V S R
gctactactttgggtgctgtgagatgcaaggtttaatttatgcagttggaggaatcagcaatgaggggct
Y Y F G C C E M Q G L I Y A V G G I S N E G L
agagctccgttcctttgaggtttatgatccactttccaagcgctggtctccacttcctcctatgggaacc
E L R S F E V Y D P L S K R W S P L P P M G T
agaagagcgtatcttggggtggcagcactcaatgactgcatctatgctattggaggctggaatgagacac
R R A Y L G V A A L N D C I Y A I G G W N E T Q
aagatgcccttcatactgtagaaaagtactccttcgaagaggaaaagtgggttgaagttgcttcaatgaa
D A L H T V E K Y S F E E E K W V E V A S M K
agttcctagagcaggcatgtgtgctgtgacagtcaatggtcttctgtatgtctctggaggccggtcttct
V P R A G M C A V T V N G L L Y V S G G R S S
agccatgatttcttggccccaggtactttcggactcagttgaagtttacaaccctcattcagatacatgg
S H D F L A P G T F G L S *
acggaaattggtaatatgatcactagtcgttgtgaaggggtgttgctgtactgtgacagagaaggcacaa
aagcacaaggaacatttgaaaatgtaggttctgaccatttgcaaatcaaacatatatttgtgatatgatc
ttctgattcttttgagtattccatgtattgaatagatagataagtaaagagtgattttctaaaatttgaa
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FIGURE 3

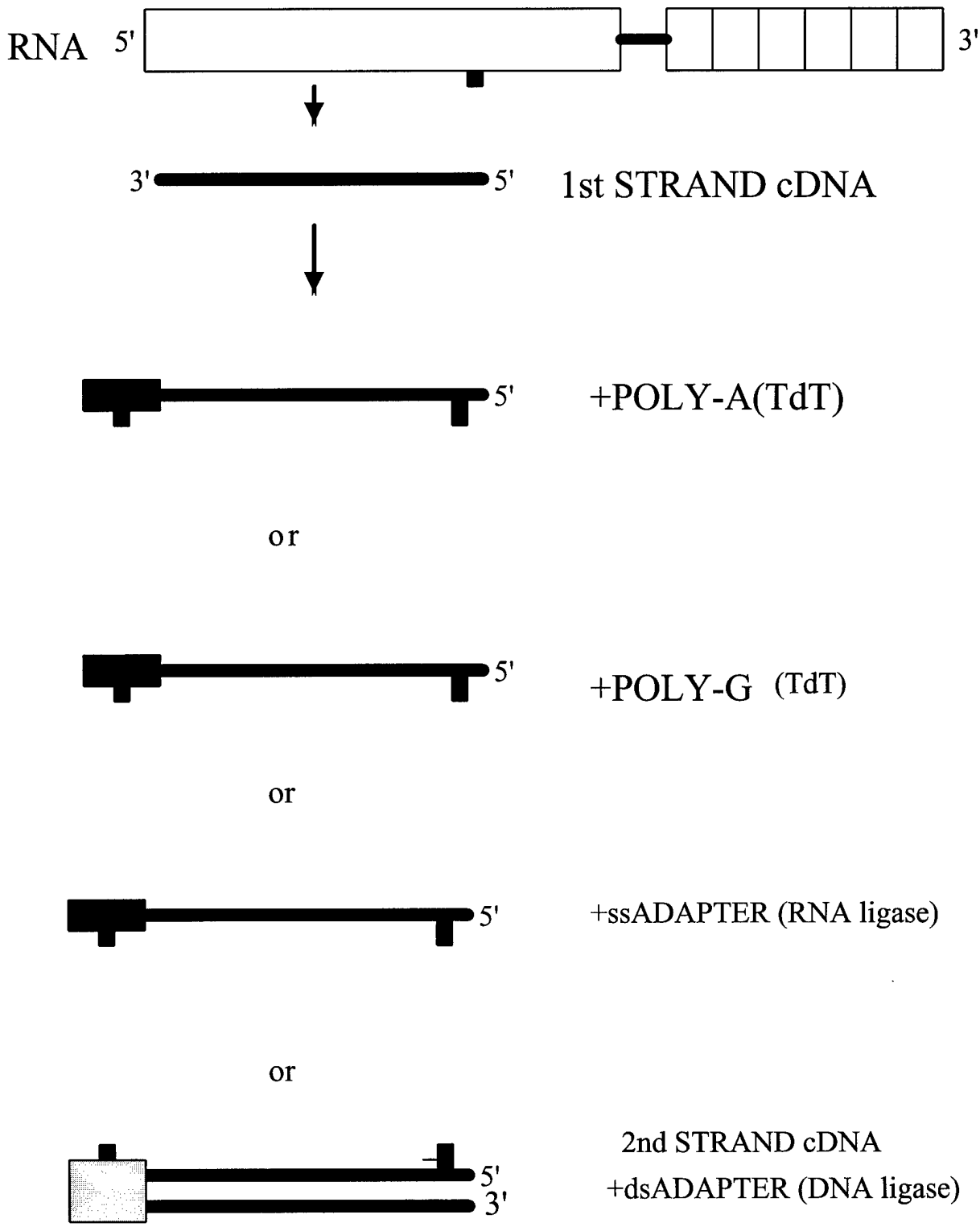
RNA
5'
3'
3'
5'
1st STRAND cDNA
5'
+POLY-A(TdT)
or
5'
+POLY-G (TdT)
or
5'
+ssADAPTER (RNA ligase)
or
5'
3'
2nd STRAND cDNA
+dsADAPTER (DNA ligase)

FIGURE 4

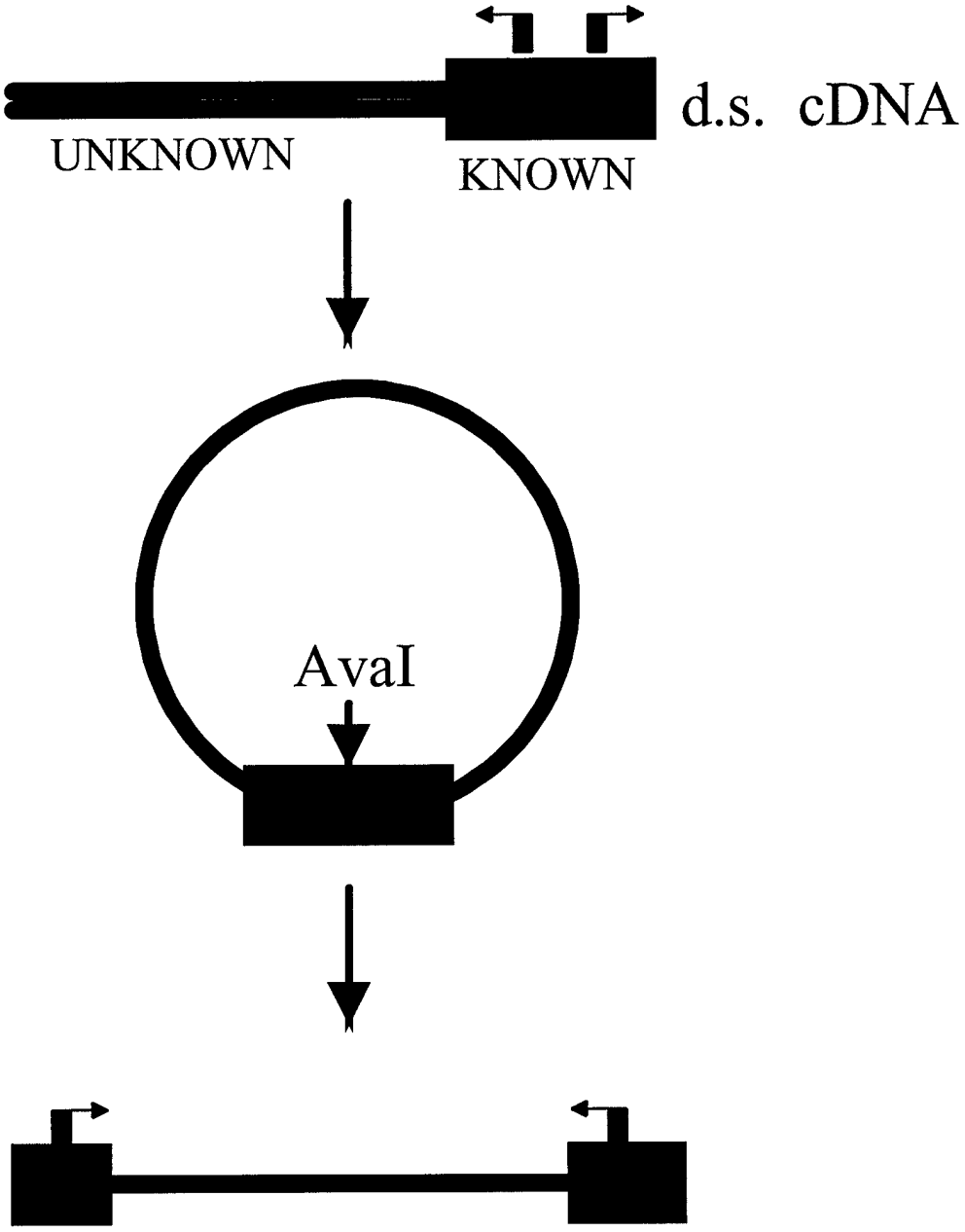

FIGURE 5

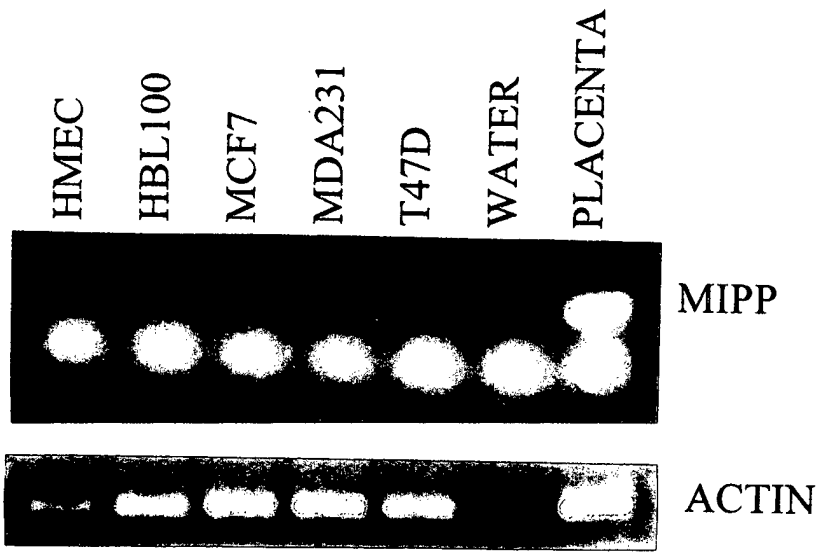

FIGURE LEGENDS:

Figure 1

A: The parallel expression of MIPP and IAPs suggests that the LTR might promote expression of MIPP in the mouse mammary tumors. To determine whether IAP LTR sequences are present in the 2.2 kb and/or 5.6 kb MIPP transcripts, RT-PCR was used. A single 3' primer was used in two sets of amplifications: one with a 5' primer from the solo IAP LTR of the MIPP gene and the other with a 5' primer downstream from the LTR. The primers are diagrammed in the top of part B and are linked to the appropriate amplification products shown in part A.

B: The predicted protein of the 1.2 kb MIPP transcript contains four *kelch* repeats, suggesting that the non-truncated transcripts might contain a BTB/POZ domain, as do other proteins with *kelch* repeats. A degenerate primer derived from the BTB/POZ domain was used in RT-PCR with a MIPP-specific primer to amplify a 994 bp fragment from a MIPP-expressing mouse mammary tumor. Upon sequencing, this fragment was found to form a single open reading frame continuous with that of the previously known MIPP sequence. The putative protein encoded by the entire 1823 bp contains six *kelch* repeats (the typical number), and a BTB/POZ domain.

Figure 2

The sequence of mouse mammary tumor MIPP transcript(s) is shown, excluding the unknown 5' end. The longest open reading frame is translated below the nucleotide sequence. The BTB/POZ domain is underlined, and the IYALGG (*kelch*) repeats are boxed.

Figure 3

5' race strategies used to amplify the 5' ends of MIPP transcripts from BALB/c mammary tumors. The heavy black lines represent the cDNA, the RNA is shown at the top of the figure in open boxes, small black boxes represent RT or PCR primers, and large black or shaded boxes represent the appropriate tail or adapter oligonucleotide (see text for details). (Terminal deoxyribonucleotidyl transferase = TdT; ss =single stranded; ds =double stranded)

Figure 4

An inverse PCR strategy was used to clone the 5' end of MIPP RNAs in mouse mammary carcinomas. Double stranded (d.s.) cDNA was circularized by blunt-ended ligation, and linearized by digestion with AvaI. This results in the inversion of opposite facing PCR primers (small boxes with arrows), so that the unknown 5' end of the cDNA is now between inward-facing primers.

Figure 5

RT-PCR analysis was used to determine whether IPP, the human homolog of MIPP, is expressed in a culture of normal, mortal mammary epithelial cells (HMEC), a benign, immortal human mammary epithelial cell line (HBL100), or in human breast cancer cell lines (MCF7, MDA-MB-231, T47D). Primers were derived from a 280 bp region in which the human and mouse genes share 83% homology at the nucleotide level. BALB/c mouse placenta was included as a positive control, and a reaction was done with no cDNA as a negative control (WATER). Actin was used as an internal control of reverse transcription and amplification.